Controlling Physico-Chemical Properties of Macrocyclic Peptides with Target-Engaging Crosslinks

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Appendices

Summary

Macrocyclic peptides typically exhibit improved physico-chemical properties such as target affinity, proteolytic stability and cell permeability when compared to their linear analogs. The rising number of design strategies and applications of macrocyclized peptides led to an increased knowledge about structure-property- and structure-activity-relationships. In this regard, a few reported examples of side chain-to-side chain cyclized peptides revealed crosslink-mediated target interactions, thereby rendering the tether a suitable site for modifications to regulate molecular properties and biological activities. In this thesis, cyclic peptides were developed with emphasis on chemical modifications at the introduced hydrocarbon linkers aiming to address protein targets with biological relevance.

Chapter 1 (*Introduction*) overviews the potential of peptides as modulators for biological targets and their advancement through macrocyclization. Various methods are characterized employing one and two components for the establishment of covalent tethers that are designed to stabilize α-helical peptides. Among these so-called stapled peptides, ligands with crosslink-mediated target interactions represent a particularly interesting class, as they offer the opportunity for further structure-guided

modifications. Examples of such peptides known in literature are briefly discussed regarding their discovery, design and relevance of the crosslink in target binding.

In **Chapter 2** (*Peptide-Based Ligands for the Inhibition of the Human Adaptor Protein 14-3-3*), the reported macrocyclic peptide $\beta_{SS}12$ with high affinity towards 14-3-3 was further developed aiming for increased drug-like properties. Truncation studies to reduce the molecular flexibility resulted in tremendous loss of binding affinity. However, a molecular docking approach using $\beta_{SS}12$ full length as a starting point yielded a peptide, which contains two additional non-natural amino acid residues and reveals an improved affinity, proteolytic stability and biological activity.

Based on the known 14-3-3–binding macrocycles $\beta_{SS}12$ and $\beta_{RS}8$, derivatives harboring an alkyne moiety at different positions within the initially saturated crosslink were investigated regarding their target affinity in **Chapter 3** (*Constraining an Irregular Peptide Secondary Structure through Ring-Closing Alkyne Metathesis*). The macrocycle with the strongest 14-3-3–interaction was applied to co-crystallization and thereby provided the first reported crystal structure of an alkyne-crosslinked peptide in complex with its target protein. Additionally, the structure elucidation confirmed the stabilization of an irregular peptide conformation by the alkyne-tether.

In **Chapter 4** (*Adapting Free Energy Perturbation Simulations for Large Macrocyclic Ligands: How to Dissect Contributions from Direct Binding and Free Ligand Flexibility*), a minimal 14-3-3–binding sequence has been derived from a crosslinked peptide. In a structure-guided optimization approach, this truncated peptide was used as starting point for the design of derivatives with varying Cα substitution pattern at the crosslinking amino acids. Both affinities and lipophilicities of corresponding peptides revealed a strong dependency on the relatively small Cα modifications (H, Me, Et). These observations were rationalized by free energy perturbation calculations and molecular dynamics simulations, which allowed to dissect the measured differences in binding affinity to contributions of direct interactions and conformational aspects. One high affinity binder has been co-crystalized with 14-3-3 visualizing the anticipated binding mode, whereas both the hydrocarbon crosslink and the introduced ethyl group occur to be involved in target engagement.

Encouraged by the considerable effect of small chemical modifications observed in Chapter 4, the introduction of a double bond into the crosslink has been investigated in **Chapter 5** (*Binding Affinity and Lipophilicity of Macrocyclic Peptides Depend on the Double Bond Conformation and Position within a Hydrocarbon Crosslink*) as an additional structural variation. Herein, fluorescence polarization assays of crosslinked

peptides with an olefin moiety showed clear trends in target affinities depending on the Cα substitution pattern, double bond position and configuration. For selected peptides, ^{1}H NMR measurements were performed to identify *E*- and *Z*- configured double bonds thereby revealing structural insights.

Chapter 6 (*Rational Design of Mechanism-Based Inhibitors for CYP121 from Mycobacterium tuberculosis*) focused on the cytochrome P450 protein CYP121, which appears to be a promising target for the development of anti-tuberculosis drugs. First, the natural substrate cYY was used to identify cognate redox partners of CYP121 required for efficient cYY metabolism. Inspired by the substrate structure, a series of electrophile-containing derivatives has been synthesized and evaluated regarding their ability to serve as a mechanism-based inhibitor for CYP121. Alkyne-modified analogs maintaining the cYY substitution pattern and stereochemistry revealed a good target affinities, inhibitory activities, low off-target effects and even indicate an irreversible, covalent mechanism of inhibition and a potential antimicrobial activity in *Mycobacterium tuberculosis* cells.

Taken together, work presented in this thesis can be seen as fundament for future research on macrocyclic peptides aiming to advance crosslink-mediated interactions and to apply computational methods rationalizing physico-chemical properties of flexible ligands. In addition, exoenzyme S-derived, crosslinked peptides established herein can be used as molecular probes for 14-3-3-dependent pathways, while the alkyne-containing cYY analog appears to be a promising starting point for the development of a novel strategy to treat tuberculosis disease.